Journal of Biogeography (J. Biogeogr.) (2013) 40, 1240–1251

ORIGINAL ARTICLE

Co-invasion of South African ecosystems by an Australian legume and its rhizobial symbionts

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ABSTRACT

Aim To determine and compare the taxonomic identity and diversity of root nodule and rhizospheric microbial symbionts associated with *Acacia pycnantha* Benth. in its native (Australian) and invasive (South African) ranges, and to establish whether these associations are general or host specific.

Location The native range of *A. pycnantha* in Australia and invasive ranges in South Africa and Western Australia.

Methods Bacteria were isolated from root nodules collected from 18 populations of *A. pycnantha*. Repetitive element polymerase chain reaction (REP-PCR) fingerprinting was used to assess overall bacterial diversity and clustering. Molecular phylogenies for a subset of isolates representing major REP-PCR clades were reconstructed using maximum parsimony and Bayesian phylogenetic analyses of the nuclear 16S–23S rRNA intergenic spacer (IGS), 16S rRNA, and the symbiotic *nodA* genes.

Results Twelve clusters were identified from the REP-PCR analysis; 11 included isolates from both the native range in Australia and invasive range in South Africa, while one cluster comprised only Australian isolates. Six rhizobial species were found in association with *A. pycnantha*: *Bradyrhizobium japonicum*, *Rhizobium gallicum, R. lusitanum, R. miluonense*, *R. multihospitium* and *R. tropici*. We also identified three plant-growth promoting bacteria isolated from root nodules of *A. pycnantha*: *Burkholderia caledonica*, *B. graminis* and *B. phytofirmans*. Phylogenetic analysis of the IGS gene retrieved clades containing symbionts from both Australia and South Africa while others comprised only South African taxa, suggesting the introduction of bacterial lineages from Australia to South Africa. Our phylogeographic analysis of the *nodA* gene confirmed that *A. pycnantha* was co-introduced with its symbionts to South Africa.

Main conclusions *Acacia pycnantha* is a promiscuous legume, associated with at least six different rhizobial symbionts, and forms associations with plant-growth promoting rhizosphere bacteria from the genus *Burkholderia*. In the invasive range of *A. pycnantha* in South Africa, nodules contained some symbionts of South African origin while other symbionts appear to have been co-introduced from Australia. *Acacia pycnantha* is associated with a wider suite of symbionts in its invasive than native range.

Keywords

***Acacia pycnantha*, Australia, biological invasions, co-introduction hypothesis, generalist, host-jumping hypothesis, mutualisms, plant growth promoters, rhizobia, South Africa.**

doi:10.1111/jbi.12091

Journal of Biogeography

INTRODUCTION

The establishment of introduced plants is dependent on a variety of factors, including direct interactions with mutualists, such as pollinators and soil microbiota (Richardson *et al.*, 2000). A growing body of evidence suggests that mutualisms between introduced plants and soil microbiota could serve as major drivers for plant invasions by improving the host's nutrient status, e.g. mycorrhizal fungi and nitrogen-fixing bacteria (rhizobia) (Reinhart & Callaway, 2006). Similarly, other beneficial microbial associations can promote plant growth by inducing metabolic processes that counter negative feedbacks brought about by biotic and abiotic stress (Compant *et al.*, 2008).

In general, mutualisms between invasive plants and rhizobia have been shown to increase plant biomass and to improve establishment success (Weir *et al.*, 2004). For example, invasive *Acacia longifolia* in Portugal, grown in soils that were collected from sites with established legume populations (*Acacia longifolia*, *Cytisus grandiflorus* and *Ulex europaeus*), produced higher above-ground biomass and nodule densities than plants grown in soils collected from established *Pinus pinaster* stands (Rodríguez-Echeverría *et al.*, 2009). High levels of nodulation in the invasive range are indicative of the functionality and the importance of mutualisms during spread and invasion processes (Parker, 2001). For example, in the USA, inoculation with *Bradyrhizobium* associated with invasive *Cytisus scoparius* more than doubled the average plant biomass while non-inoculated plants without root nodules had significantly lower biomass (Parker *et al.*, 2006).

The origin(s) of nitrogen-fixing symbionts utilized by invasive legumes in their new ranges has been a subject of discussion in the invasion literature (Weir *et al.*, 2004; Chen *et al.*, 2005; Parker *et al.*, 2007; Wei *et al.*, 2009; Rodríguez-Echeverría, 2010; Porter *et al.*, 2011; Rodríguez-Echeverría *et al.*, 2011; Crisóstomo *et al.*, 2013). Many successful invasive legumes form new mutualisms with bacteria found in the introduced environment (host-jumping hypothesis). However, symbionts can also be co-introduced with host plants, either directly as inoculants for agroforestry species (Marques *et al.*, 2001) or indirectly by hitchhiking on introduced plant material (co-introduction hypothesis) (Weir *et al.*, 2004; Porter *et al.*, 2011). Some invasive legumes form a variety of different mutualisms, and can conform to both hypotheses. For example, invasive populations of *Acacia longifolia* and *A. saligna* from Portugal, *Medicago polymorpha* from California, USA, and *Acacia decurrens* from New Zealand, all recruit co-introduced bacterial symbionts (Weir *et al.*, 2004; Rodríguez-Echeverría, 2010; Porter *et al.*, 2011; Crisóstomo *et al.*, 2013) and some Australian acacias are able to recruit novel symbionts when grown in non-native soils (Birnbaum *et al.*, 2012).

This mixing of symbionts from different origins can have several consequences. In some instances, subsequent conjugation between different bacterial strains of different origins can lead to novel genetic combinations and may even enhance invasiveness (Menna & Hungria, 2011). Alternatively, co-introduced bacteria may represent a preferred symbiotic lineage or be a different species, which can outcompete local microbiota, resulting in multiple invasions, both above and below ground (Rodríguez-Echeverría, 2010). However, different rhizobial strains usually vary in effectiveness, with co-evolved associations being the most effective. Therefore, while the ability of an invader to form mutualisms with a larger variety of rhizobia (symbiotic promiscuity) significantly improves invasion potential and success (Weir *et al.*, 2004; Rodríguez-Echeverría *et al.*, 2011) through host-jumping, these may not be the most effective associations. Generally, promiscuous host plants also tend to be more effective in fixing nitrogen, implying that in new environments where host plants have a choice of symbiotic partners, mutualistic interactions are likely to favour generalists and exclude specialists (Wilkinson & Parker, 1996).

In addition to housing nitrogen-fixing bacteria, some legumes can also interact with other forms of plant-growth promoting microbes in their root nodules. For example, co-inoculation of soya bean with *Bradyrhizobium japonicum* and the plant-growth promoting bacterium *Serratia proteamaculans* increases the onset of nitrogen fixation, percentage plant nitrogen produced, and plant protein content (Dashti *et al.*, 1998). Similarly, endophytic plant-growth promoting bacteria and nitrogen-fixing *Rhizobium* species were found to work in synergy to promote nitrogen fixation efficiency in *Lens culinaris* (Veena & Poonam, 2011).

Not surprisingly, legumes are often over-represented among invasive plant taxa (Daehler, 1998) with some taxa emerging as model systems within invasion biology, e.g. Australian acacias (Richardson *et al.*, 2011). Many species of acacias have been moved around the world for various purposes, with many records of invasiveness. In particular, in South Africa dense monospecific stands of acacias cover tens of thousands of hectares, with substantial impacts, including changes in the soil microbial structure as a result of increased soil nitrogen (Gaertner *et al.*, 2009; Le Maitre *et al.*, 2011). The success of *Acacia* invasions globally has been attributed to a number of factors such as repeated introductions, introductions of large seed volumes, and concerted breeding efforts by scientists (Le Roux *et al.*, 2011). However, it is also likely that mutualisms, such as the legume–rhizobial symbioses, play an important role in the establishment and invasion success of acacias (Rodríguez-Echeverría *et al.*, 2011). Despite the huge introduction efforts and successful invasions by Australian acacias in South Africa, little is known of the diversity and origin of rhizobia nodulating these species and their role in the invasion process (but see Rodríguez-Echeverría *et al.*, 2011). Here, we use *Acacia pycnantha* Benth., a tree native to eastern and south-eastern Australia and invasive in the Eastern and Western Cape Provinces of South Africa, to understand the diversity and role of rhizobial symbionts in its invasion success. Specifically we aimed to: (1) determine the taxonomic diversity

of rhizobia associated with root nodules of *A. pycnantha* in both native and invasive ranges; (2) determine whether rhizobial associates of invasive *A. pycnantha* in South Africa follow the co-introduction, host-jumping hypothesis, or both; and (3) determine the level of promiscuity of *A. pycnantha*–rhizobial associations.

MATERIALS AND METHODS

Root nodule collection

During 2009 root nodules were collected from *A. pycnantha* plants from 10 sites from throughout its native range in Australia. We also collected plants from seven sites in South Africa and one site in Western Australian (Table 1), representing its introduced ranges. At each site, five root nodules were sampled from 10 individual plants. Root nodules from a single host plant were treated as one unit and preserved in the same vial containing silica gel. For phylogenetic analyses we sequenced only one bacterial isolate/host plant because most bacteria retrieved represented *Bradyrhizobium* spp. (see Results) and all bacteria collected from single hosts appeared to be the same species based on morphology and growth rates. We therefore focused our phylogenetic analyses to include more sites over wider geographic regions rather than more nodules/individuals.

Isolation and culturing of rhizobia

Dried root nodules were washed thoroughly using tap water to remove all soil particles. Nodules were transferred to sterile water and stored in a refrigerator at 4 °C overnight to rehydrate (Burdon *et al.*, 1999). Rehydrated nodules were surface-sterilized in 70% ethanol for 30 s followed by 3.5% sodium hypochlorite solution for 5 min. Sterilized nodules were washed at least five separate times in sterile distilled water. The surface sterilized root nodules were then crushed in 300 μL sterile distilled water using a sterilized toothpick. 50 μL of the turbid suspension was diluted and streaked on yeast manitol agar (YMA) medium (Vincent, 1970) containing Congo Red. Plates were incubated at 28 °C and examined for bacterial growth after 3–10 days. Well-separated single colonies were restreaked on to fresh plates until pure cultures were obtained. Colony purity was ascertained using Gram staining and light microscopy.

Genomic DNA isolation

Genomic DNA was extracted by heating bacterial cells in a lysis buffer (1 × TE: TrisHCl and EDTA) for 5 min at 95 °C followed by centrifuging cell lysates for 2 min. The DNA-containing supernatant was collected and washed with chloroform (Parker *et al.*, 2007). All DNA concentrations were quantified by spectroflourimetry and high quality extractions stored at −20 °C until further use.

DNA fingerprinting

To obtain an overall idea of bacterial diversity among isolates a preliminary repetitive element polymerase chain reaction (REP-PCR) fingerprinting was performed using a fluorescently labelled enterobacterial repetitive intergenic consensus primer (ERIC1) (5′-ATG TAA GCT CCT GGG GAT TCA-3′) and a BOX primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) (Versalovic *et al.*, 1994). The REP-PCR was carried out in 20 μL reactions containing 5 pmol of each primer, 25 ng template bacterial DNA, 0.4 μL of 10 mM dNTPs, 5 U taq (Kapa Biosystems, Boston, MA, USA), 10 mg mL^{-1} bovine serum albumen (BSA) and 4 μL of 5 × buffer. PCR amplifications were performed in an automated thermocycler with initial denaturation of 95 °C for 2 min, followed by 40 cycles of denaturation (94 °C for 60 s, 53 °C for 60 s, 65 °C for 8 min) and a final extension temperature of 65 °C for 8 min. PCR products were separated on 2% agarose gels to confirm successful amplification prior to genotyping (Versalovic *et al.*, 1994). PCR fragments were separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using GeneScan™-500 (-250) as an internal size standard (Applied Biosystems). Allele sizes were visualized and scored as presence or absence of amplicons using GeneMarker® 1.95 (SoftGenetics LLC®, State College, PA, USA). A binary matrix of presence and absence data was generated for all amplified fragments. These data were used to reconstruct a dendrogram using a neighbour-joining distance algorithm as implemented in PAUP* 4 (Swofford, 2002).

PCR conditions and DNA sequencing

Distinct clusters from the ERIC and BOX fingerprinting tree were visually identified. At least two representative isolates were chosen from each cluster and sequenced using the 16S–23S rRNA intergenic spacer region (IGS). IGS amplification was carried out using the primers FGPS 1490-72 (5′-TGC GGC TGG ATC CCC TCC TT-3′) and FGPL 132'-38 (5′-CCG GGT TTC CCC ATT CGG-3′) (Romdhane *et al.*, 2005). Each 50 μL reaction contained 5 pmol of each primer, 0.5 μL of 20 mM dNTPs, 10 × buffer, 1.5 mM magnesium chloride and 0.5 μL Super-Therm polymerase JMR-801 (5 U μL^{-1}) (Roche, Mannheim, Germany). The following PCR cycle was used: 94 °C for 5 min denaturation followed by 30 cycles of (94 °C for 30 s, 58 °C for 30 s annealing and 72 °C for 60 s) followed by a final extension step at 72 °C for 7 min and a final holding temperature of 15 °C. A preliminary BLAST search of the 16S–23S IGS sequences was performed to compare sequences obtained against the NCBI database. Sequences that had a similarity index below 96% could not be used to reliably identify isolates to species level were reamplified with the 16S rRNA primers, 16SA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 16SB (5′-AAG GAG GTC ATC CAG CC-3′) (Weisburg *et al.*, 1991). For the latter, PCR reactions were carried out in 50 μL reactions containing

Table 1 Rhizobial strains used in the phylogenetic analyses of root nodule and rhizospheric microbial symbionts associated with *Acacia pycnantha* in its native (Australian) and invasive (South African and Western Australian) ranges.

					GenBank accession numbers		
Sample ID	Country	Locality	Latitude	Longitude	16S–23S IGS	16S rRNA	*nodA*
JNR1	SA	Caledon	−33.10701	19.29755	NA	KC207926	NA
JNR2	SA	Caledon	−33.10701	19.29755	HQ895988	NA	NA
JNR5	SA	Caledon	−33.10701	19.29755	HQ895989	NA	NA
JNR6	SA	Caledon	−33.10701	19.29755	HQ895990	NA	KC297661
JNR8	SA	Caledon	−33.10701	19.29755	HQ895991	NA	NA
JNR20	SA	Humansdorp	−34.03989	24.78687	NA	KC207924	NA
JNR22	SA	Humansdorp	−34.03989	24.78687	NA	KC207922	NA
JNR24	SA	Humansdorp	−34.03989	24.78687	NA	KC207913	NA
JNR25	SA	Humansdorp	−34.03989	24.78687	NA	KC207925	NA
JNR26	SA	Humansdorp	−34.03989	24.78687	NA	KC207920	NA
JNR27	SA	Humansdorp	−34.03989	24.78687	NA	KC207923	NA
JNR28	SA	Humansdorp	−34.03989	24.78687	NA	KC207911	NA
JNR30	SA	Humansdorp	−34.03989	24.78687	HQ895993	NA	KC297663
JNR31	SA	Wolseley	−33.34012	19.16109	HQ895994	NA	KC297664
JNR32	SA	Wolseley	−33.34012	19.16109	KC207904	NA	KC297665
JNR35	SA	Wolsely	−33.34012	19.16109	HQ895995	NA	KC297666
JNR37	SA	Wolsely	−33.34012	19.16109	HQ895996	NA	KC297667
JNR42	SA	Tokai	−33.84179	18.66602	NA	KC207921	NA
JNR44	SA	Tokai	−33.84179	18.66602	HQ895997	NA	KC297668
JNR53	SA	Stellenbosch	−34.06024	18.41480	KC207906	NA	KC297669
JNR54	SA	Stellenbosch	−34.06024	18.41480	HQ895998	NA	KC297662
JNR56	SA	Stellenbosch	−34.06024	18.41480	NA	KC207917	NA
JNR57	SA	Stellenbosch	−34.06024	18.41480	KC207905	NA	KC297670
JNR138	SA	Piketberg	−32.80084	18.71501	HQ896012	NA	NA
JNR62	AUS	Esperance	−34.31586	118.79919	NA	NA	KC297671
JNR63	AUS	Esperance	−34.31586	118.79919	KC207903	NA	NA
JNR65	AUS	Esperance	−34.31586	118.79919	NA	KC207930	NA
JNR67	AUS	Esperance	−34.31586	118.79919	NA	KC207933	NA
JNR69	AUS	Esperance	−34.31586	118.79919	HQ896000	NA	NA
JNR71	AUS	Esperance	−34.31586	118.79919	KC297672	NA	KC297672
JNR78	AUS	Melrose	−32.78187	138.1973	KC207902	NA	NA
JNR80	AUS	Melrose	−32.78187	138.1973	HQ896003	NA	KC297674
JNR83	AUS	Melrose	−32.78187	138.1973	HQ896004	NA	KC297675
JNR85	AUS	Melrose	−32.78187	138.1973	KC207899	NA	KC297676
JNR86	AUS	Frances	−36.77054	141.18135	NA	KC207912	NA
JNR89	AUS	Frances	−36.77054	141.18135	HQ896005	NA	KC297680
JNR93	AUS	New South Wales	−35.99273	143.76538	KC207900	NA	KC297677
JNR94	AUS	New South Wales	−35.99273	143.76538	NA	KC207932	NA
JNR98	AUS	Mt Jerrabomberra	−35.36866	149.20332	NA	NA	KC297678
JNR100	AUS	Mt Jerrabomberra	−35.36866	149.20332	NA	KC207910	NA
JNR101	AUS	Mt Jerrabomberra	−35.36866	149.20332	KC207901	NA	KC297679
JNR104	AUS	Natimuk	−36.00409	143.76041	NA	KC207916	NA
JNR109	AUS	Natimuk	−36.00409	143.76041	NA	KC207919	NA
JNR112	AUS	Natimuk	−36.00409	143.76041	NA	KC207931	NA
JNR117	AUS	Mt Compass	−35.40585	145.95586	NA	KC207914	NA
JNR118	AUS	Mt Compass	−35.40585	145.95586	NA	KC207928	NA
JNR120	AUS	Wagawaga	−35.21065	147.76425	NA	KC207918	NA
JNR125	AUS	Wagawaga	−35.21065	147.76425	NA	KC207909	NA
JNR126	AUS	Wagawaga	−35.21065	147.76425	NA	KC207908	NA
JNR129	AUS	Lockhart	−35.36866	146.64549	NA	KC207907	NA
JNR131	AUS	Lockhart	−35.36866	146.64549	NA	KC207915	NA
JNR132	AUS	Lockhart	−35.36866	146.64549	NA	KC207927	NA
JNR135	AUS	Lockhart	−35.36866	146.64549	NA	KC207929	NA

AUS, Australia; SA, South Africa; IGS, intergenic spacer; NA, not applicable.

5 pmol of the forward and reverse primers, 1 μL of 20 mM dNTP mix, 5 μL of 10 × buffer, 1.5 mM magnesium chloride, 1 μL BSA and 24.5 μL water. The following cycle was used: an initial denaturation of 95 °C for 2 min followed by 30 cycles of (95 °C for 30 s, 54 °C for 30 s annealing and 72 °C for 4 min) followed by a final extension step of 72 °C for 20 min and a holding temperature of 15 °C.

The *nodA* gene, which is commonly used as a biogeographic marker in rhizobial phylogenetics (Rodríguez-Echeverría, 2010), was amplified for isolates that were identified as *Bradyrhizobium* spp. using the primers TSnodD1-1C (5′- CAG ATC NAG DCC BTT GAA RCG CA-3′) and TSnodB1 (5′- AGG ATA YCC GTC GTG CAG GAG CA-3′) /TSnodA2 (5′-GCT GAT TCC AAG BCC YTC VAG ATC-3′) (Moulin *et al.*, 2004). The following cycle was used: 94 °C for 2 min, followed by 30 cycles of (95 °C for 30 s, 56 °C/ 55 °C for 30 s, 72 °C for 45 s) and a final extension temperature of 72 °C for 7 min (Rodríguez-Echeverría, 2010). The *nodA* gene (350 bp fragment) was sequenced for all isolates included in the 16S rRNA analysis and also to check whether taxa identified as *Burkholderia* (see Results) were actually nodulating bacteria. *Burkholderia terricola* was used as a positive control for the latter. For *Burkholderia* accessions, the *nodA* gene was amplified using the primers nodA A (5′-TGG ARV BTN YSY TGG GAA A-3′) and nodB B (5′-CCR AAV SCR AAY GGV AC-3′) and the following thermocycle: 94 °C for 2 min followed by 35 cycles of (94 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s) and a final extension temperature of 72 °C for 7 min (Chen *et al.*, 2005).

Molecular characterization of isolates and phylogenetic analysis

A BLAST search of the 16S–23S IGS, 16S rRNA and the *nodA* gene sequences was performed to compare sequences obtained in this study against existing data available in GenBank (http://blast.ncbi.nlm.nih.gov). Australian and South African sequences available from GenBank that revealed high similarities to those obtained here (96–100% similarity) were also included in the reconstruction of phylogenies. Datasets for all gene regions were aligned using Clustal W (Thompson *et al.*, 1994) and manually edited in BioEdit 7.0.5.3 (Hall, 1999). All three gene phylogenies were reconstructed using maximum parsimony as implemented in PAUP* 4 with the heuristic search options (Swofford, 2002) and Bayesian inference in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). MrBayes was run for two million generations and trees sampled every 100 generations for all the three genes. Confidence in tree topologies for maximum parsimony was determined using 1000 bootstrap replicates while Bayesian posterior probabilities were determined with the Bayesian Markov chain Monte Carlo method implemented in MrBayes 3.1.2. Prior to analyses, the best fit model for evolution for each of the datasets was determined using the Akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posoda & Crandall, 1998). The model of evolution used to reconstruct the *Bradyrhizobium* phylogeny was the HKY + I + G model, while the TrN + I + G and the TVM + G models were used for the 16S rRNA and *nodA* phylogenies, respectively. Because the latter two models are not implemented in MrBayes, we used the GTR + G model.

RESULTS

From 180 collected root nodules, 125 produced colonies resembling rhizobia. Among these there was a mixture of fast-growing and slow-growing bacteria. Most colonies from native range regions were fast growing (*c.* 63%) while most colonies from invasive ranges were slow growing (*c.* 80%).

Neighbour joining analysis of the REP-PCR and ERIC-PCR fingerprints revealed 12 distinct genetic clusters of bacterial symbionts (results not shown). Overall, Australian (native) and South African (invasive) isolates were found in most clusters retrieved, with the exception of one cluster that contained only Australian isolates.

16S–23S rRNA – *Bradyrhizobium*

All sequences of slow-growing bacteria showed high similarity (> 96%) to accessions of *B. japonicum* lodged in GenBank. All the trees obtained from the two different analyses (Bayesian and maximum parsimony) retrieved similar topologies so only the Bayesian tree is shown here (Fig. 1). All analyses separated the *Bradyrhizobium* isolates into nine well-supported and distinct clades (Fig. 1). Clade 6 consisted of isolates with Australian origins, while clade 2 and clade 9 consisted of accessions collected from *A. pycnantha* from both native and invasive ranges. Clade 1 and Clade 5 consisted of isolates that were unique to the invasive range in South Africa. Most of the Portuguese isolates that had an Australian origin (based on the *nodA* and *nifD* genes, see Rodríguez-Echeverría, 2010) were found in cluster seven.

nodA

Partial *nodA* DNA sequences obtained in this study, and additional data for *Bradyrhizobium* spp. isolated from root nodules of different species in Australia and southern Africa (Botswana, South Africa and Zimbabwe) obtained from GenBank, were used for phylogenetic reconstruction. Two geographically distinct clades were resolved in the analysis (Fig. 2). Clade 1 consisted of exclusively southern African accessions (isolated from root nodules of *Arachis hypogaea* and *Vigna unguiculata*), while Clade 2 included mostly Australian accessions and isolates from South Africa (invasive *A. pycnantha* and *A. decurrens*). All invasive *A. pycnantha* isolates from South Africa grouped within the Australian clade.

16S rRNA

All of the fast-growing bacteria sequenced using the 16S–23S rRNA IGS region showed low overall similarity (< 96%) to

Figure 1 Bayesian tree of *Bradyrhizobium japonicum* symbionts associated with *Acacia pycnantha* based on the 16S–23S rRNA intergenic spacer (IGS) gene. The tree also includes reference bacterial strains isolated from various legume species, as indicated. Nodal support is given as posterior probability values. The scale bar represents the number of substitutions per site. Geographical origins are abbreviated: AUS, Australia; PORT, Portugal; SA, South Africa. Please note that, despite being collected in Portugal, accessions labelled with asterisks are thought to be of Australian origin based on a previous phylogeographic study (Rodríguez-Echeverría, 2010).

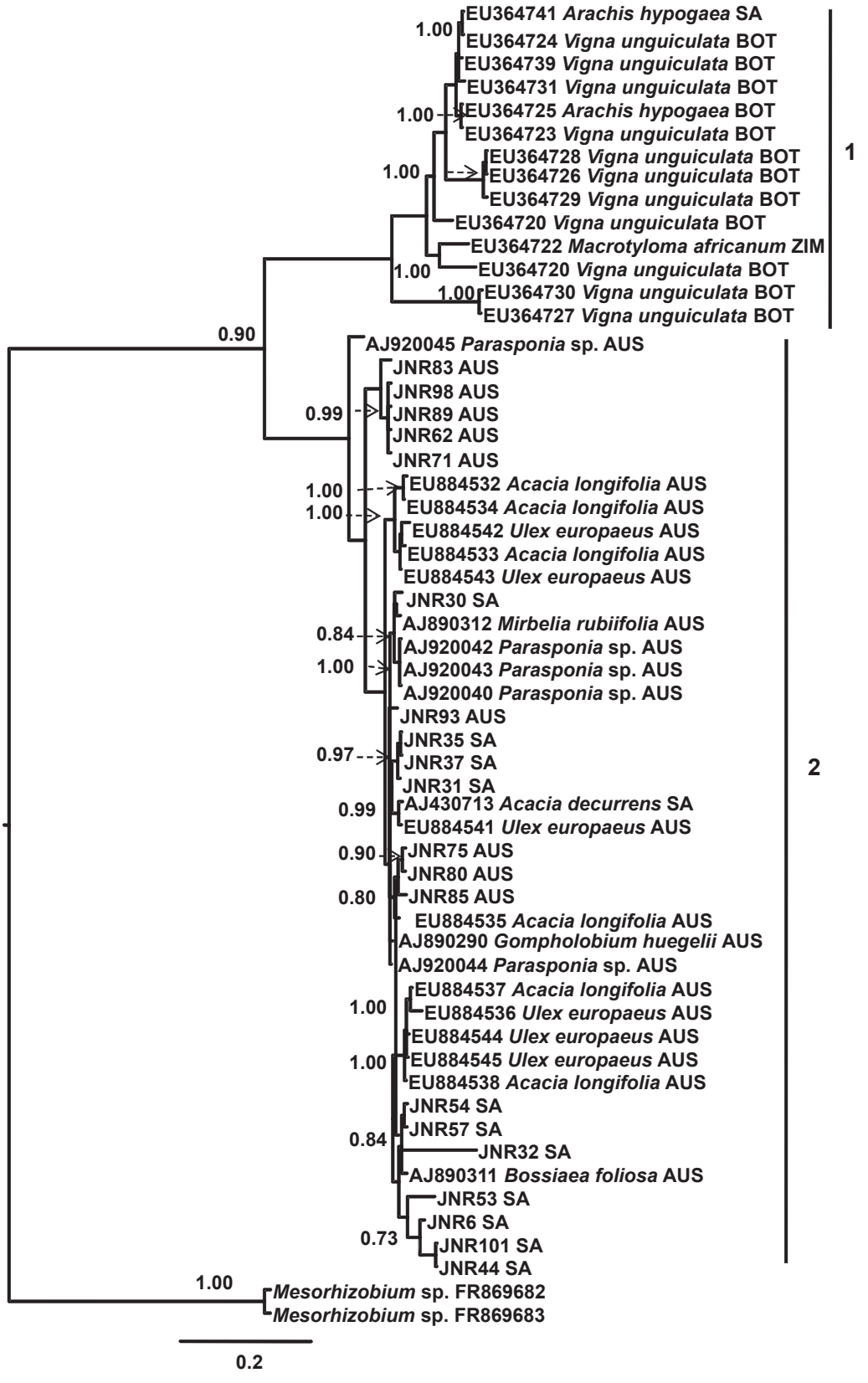

Figure 2 Bayesian tree based on the *nodA* gene of *Bradyrhizobium japonicum* symbionts associated with *Acacia pycnantha*. The tree also includes reference bacterial strains isolated from various legume species, as indicated. Posterior probability values > 80% are indicated. The scale bar represents the number of substitutions per site. Geographical origins are abbreviated: AUS, Australia; BOT, Botswana; SA, South Africa; ZIM, Zimbabwe.

any accessions deposited in GenBank. Re-sequencing these fast-growing isolates with the 16S rRNA primers yielded sequences with a high similarity index to sequences contained in GenBank and identified fast-growing bacteria as similar to *Burkholderia*, *Rhizobium* and *Mesorhizobium* species. The 16S rRNA phylogeny retrieved two major clades with one belonging to the alpha proteobacteria (*Mesorhizobium* and *Rhizobium*) and the other belonging to the beta proteobacteria (*Burkholderia*) (Fig. 3). In South Africa, isolates resembling *Mesorhizobium* spp., *Rhizobium miluonense*, *R. multihospitium* and *R. tropici* were retrieved, while in Australia *R. gallicum* and *R. lusitanum* were most closely related to fast-growing bacteria isolated from *A. pycnantha*. In addition, rhizobia closely related to *R. cellulolyticum* and *R. yanglingense* (which are known to form ineffective nodules in *Phaselous vulgaris*; Tan *et al.*, 2001; García-Fraile *et al.*, 2007) were found in the root nodules of *A. pycnantha* from Australia. The 16S rRNA phylogenetic tree placed the *Burkholderia* sequences from Australia and South Africa into a single well-supported clade that included *Burkholderia caledonica, B. graminis* and *B. phytofirmans* (Fig. 3). Both South African and Australian bacteria included *B. phytofirmans* and *B. caledonica*, while a strain similar to *B. graminis* (JNR104) was found in Australia only. All three species identified here are not known to nodulate legumes or fix nitrogen and therefore amplification of the *nodA* gene (which facilitates nodulation) failed for all the isolates. To confirm this, we successfully amplified *nodA* in *B. terricola,* a known nodulating symbiont of *Virgilia oroboides* in South Africa (A. Magadlela, Stellenbosch University, pers. comm.), using the same PCR conditions and primers.

DISCUSSION

Acacia pycnantha in its invasive range in South Africa was co-introduced with at least some of its native symbionts from Australia (co-introduction hypothesis). *Acacia pycnantha* is also a highly promiscuous nitrogen fixer capable of forming mutualistic associations with a wide range of symbionts in its introduced range. It forms associations with members of the genus *Burkholderia*, although these associations probably do not result in the fixation of atmospheric nitrogen. Such promiscuity is not surprising because other closely related species such as *Acacia cyclops*, *A. saligna*, *A. melanoxylon* and *Paraserianthes lophantha* also form new rhizobial associations when cultivated in soils from their non-native ranges (Birnbaum *et al.*, 2012). *Acacia* therefore appears to consist of generalist species, which do not require specific rhizobial species to fix nitrogen outside their native range (Rodríguez-Echeverría *et al.*, 2011; Birnbaum *et al.*, 2012).

Given the pivotal role of mutualisms in invasion success (Richardson *et al.*, 2000), species with highly specialized obligate mutualistic associations are often under-represented in invasive floras, e.g. members of the Orchidaceae (Daehler, 1998). The ability of Australian acacias to nodulate and fix nitrogen must have been a substantial factor contributing to their success in South Africa's fynbos biome, which is characterized by soils that are generally poor in nutrients, especially nitrogen (Slabbert *et al.*, 2010). While it is an advantage to form these mutualisms, the ability to form mutualistic partners with a wider range of rhizobial symbionts (generalist legumes) must confer advantages to introduced legumes in their new environments. Invasive *A. pycnantha* in South Africa is a generalist species associated with a range of different and distantly related rhizobia, including *Bradyrhizobium japonicum*, *Rhizobium gallicum, R. miluonense*, *R. multihospitium* and *R. tropici*. To the best of our knowledge this is the first record of both *R. miluonense* and *R. multihospitium* associated with an Australian *Acacia*. *Rhizobium multihospitium* was isolated from several legumes in China (Han *et al.*, 2008). Similarly, *R. miluonense* was isolated from the root nodules of *Lespedeza* species in China (Gu *et al.*, 2008). Highly promiscuous legumes do not require co-introduction with compatible rhizobia from their native regions and may easily recruit novel microsymbionts in their new environments (Rodríguez-Echeverría *et al.*, 2011).

Our phylogenetic results indicate that *B. japonicum* symbionts were most likely co-introduced with *A. pycnantha* from Australia (Figs 1 & 2). Without knowledge of native legume–rhizobia associations, novel mutualisms between *A. pycnantha* and South African rhizobia remain speculative. However, the association between *A. pycnantha* and bacteria known only from Asia (*R. miluonense* and *R. multihospitium*) supports the host-jumping hypothesis. While the phylogeographic utility of the 16S–23S rRNA IGS gene region (Fig. 1) has been questioned (Rodríguez-Echeverría, 2010), the co-introduction of symbionts from Australia is further supported by our nodulation gene (*nodA*) phylogeny. Nodulation genes have been widely used and have proven to be reliable phylogeographic markers for rhizobial lineages (Weir *et al.*, 2004). Our results confirmed that *A. pycnantha* brought most, if not all, of its *Bradyrhizobium* symbionts along from Australia (Fig. 3). All the *A. pycnantha* isolates from South Africa grouped within the Australian clade (Clade 2; Fig. 2). Overall, invasive *A. pycnantha* in South Africa appears to conform to both the co-introduction and the host-jumping hypothesis. Consequently, *A. pycnantha* utilizes a wider suite of symbionts in its invasive range than its native range. This finding contrasts with previous work that showed Australian acacias to generally utilize a wider variety of mutualists in their native than invasive ranges (e.g. Weir *et al.*, 2004; Rodríguez-Echeverría, 2010; Rodríguez-Echeverría *et al.*, 2011).

In addition to the conventional rhizobial taxa found associated with *A. pycnantha* in Australia and South Africa, we also identified various *Burkholderia* taxa. The association of some beta-proteobacterial genera capable of fixing atmospheric nitrogen, including *Burkholderia,* has been known for some time (Moulin *et al.*, 2001). In particular, we isolated and identified *B. caledonica*, *B. graminis* and *B. phytofirmans* from root nodules of *A. pycnantha*. While nitrogen-fixing

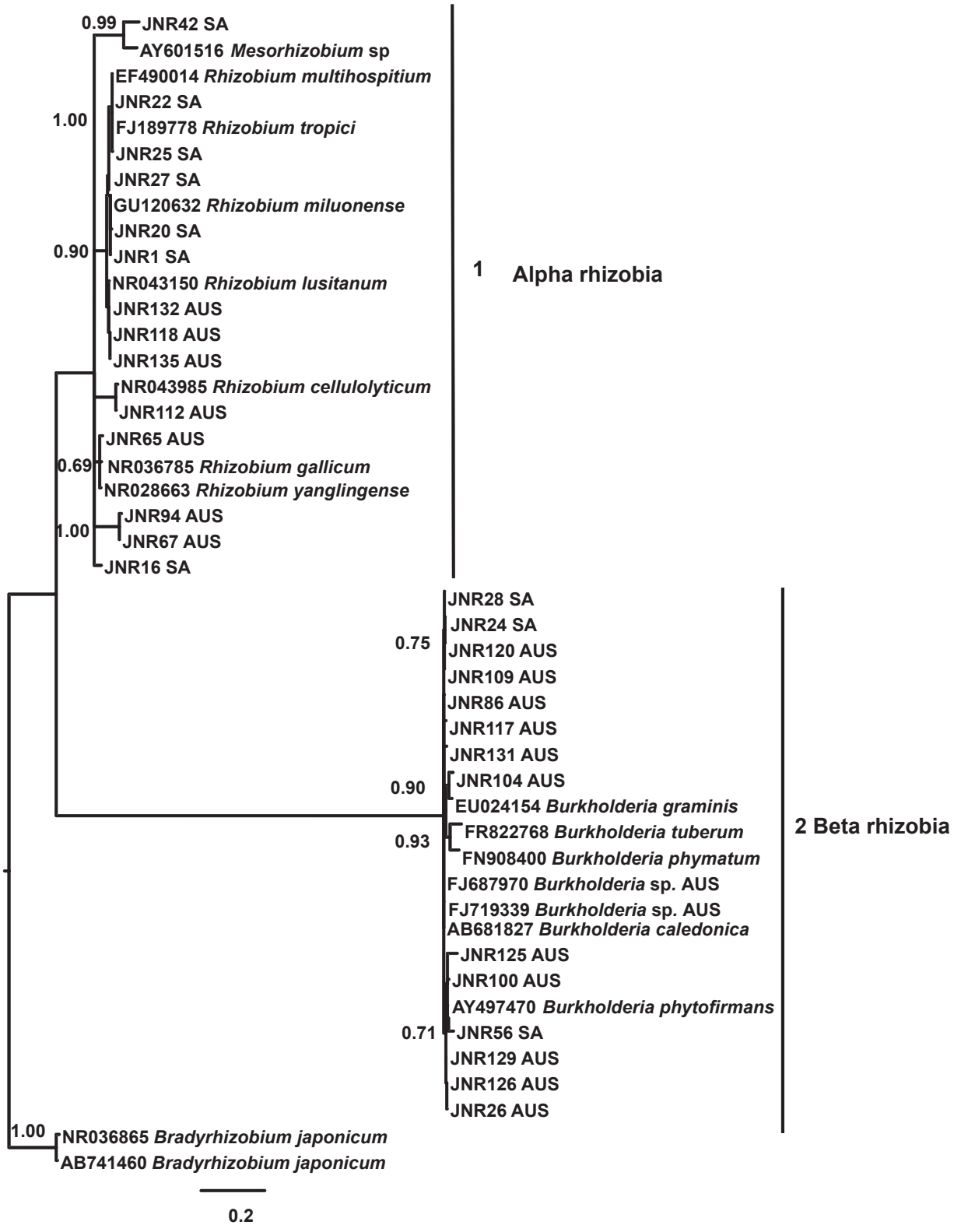

Figure 3 Bayesian tree based on the 16S rRNA gene for *Burkholderia, Rhizobium* and *Mesorhizobium* symbionts found associated with *Acacia pycnantha* in South Africa and Australia. GenBank accession numbers are given for reference taxa included from GenBank. Nodal support is given as Bayesian posterior probability values. The scale bar represents the number of substitutions per site. Geographical origins of *A. pycnantha* symbionts are abbreviated: AUS, Australia; SA, South Africa.

Burkholderia endosymbionts have been previously described from numerous South African legumes (Elliot *et al.*, 2007), and it is hypothesized that the group has a primarily Neotropical origin (Bontemps *et al.*, 2010). As far as we know, no indigenous nodulating *Burkholderia* strains are known from Australia. Numerous attempts to amplify nodulation genes (*nodA*) in these isolates failed, whereas we could successfully amplify nodulations genes from *B. terricola*, which nodulates South African legumes and fixes nitrogen (A. Magadlela, pers. comm.). Nevertheless, the frequency of *Burkholderia* strains was overall very high, and therefore not considered to be the result of contamination but is likely to reflect true symbionts. Whilst these taxa appear incapable of fixing nitrogen, the data raise the fundamental question of whether these bacteria have benefits to their hosts. Two of these species, *B. caledonica* and *B. graminis,* have been previously recorded as common rhizosphere inhabitants (Compant *et al.*, 2008), while others have been found to be specifically associated with the rhizospheres of Australian acacias (Hoque *et al.*, 2011). These rhizosphere bacteria can form beneficial associations with their hosts, although the nature of these associations remains uncertain. Plant-growth promoting rhizobacteria can colonize the plant anterior and thrive as endophytes in various plant organs without causing harm to their hosts (Compant *et al.*, 2008). These bacteria initially colonize the root surface followed by the subsequent entrance into the endorhiza mainly through the root tip, lateral root cracks or rhizodermal cells via cell wall degrading enzyme secretions (Compant *et al.*, 2008). Plant growth promoters can directly exert positive effects to plants through the synthesis of phytohormones and solubilization of inorganic phosphates and mineralization of organic phosphates (Rodríguez & Fraga, 1999).

The isolation of *B. phytofirmans* from root nodules of *A. pycnantha* from both native and invasive ranges is a significant finding. *Burkholderia phytofirmans* has been largely recognized as a plant-growth promoting bacterium. For example, associations with *B. phytofirmans* enlarge root systems with enhanced secondary roots and more root hairs and thus the opportunity to form root nodules by rhizobia. Association with *B. phytofirmans* also frequently leads to the development of more and larger leaf hairs, steadier stems, higher lignin deposits around the vascular system, larger amounts of chlorophyll, increased levels of cytokinins and phenylalanine ammonia, and pathogen resistance (Compant *et al.*, 2008). Assuming that the endophytic bacteria confer some or all of these advantages to legumes that already have the ability to overcome the negative effects caused by nitrogen stress, plant establishment and subsequent invasions will therefore benefit. However, more research is needed to understand the role of the endophytic bacteria not only in the establishment success of the genus *Acacia*, but for introduced legumes in general. This information will not only advance our understanding of the invasion dynamics of Australian acacias but may also increase the value of these species for agroforestry. To date, *Burkholderia* has been isolated from root nodules or the rhizosphere of *A. pycnantha*, *A. salicina*, *A. stenophylla* and *A. decurrens* (Menna *et al.*, 2006; Hoque *et al.*, 2011; Rodríguez-Echeverría *et al.*, 2011). We recommend that future research should focus on assessing the beneficial properties that these endophytic *Burkholderia* species may render invasive legumes.

Finally, in the South African fynbos, where *A. pycnantha* is invasive, indigenous legumes show distinct, and some even peculiar, rhizobial associations. For example, the genus *Cyclopia* is nodulated primarily by *Burkholderia* species, while some *Lotononis* species are nodulated by *Methylobacterium nodulans* (Sy *et al.*, 2001; Ardley *et al.*, 2009); both groups are very distantly related to conventional rhizobia. The diversity of rhizobial symbionts in the fynbos offers an exciting opportunity to study how the interactions between indigenous and introduced bacteria affect both native and introduced legume species, a largely unexplored topic. A comprehensive study of symbiotic genes will allow for a detailed framework on how lateral gene transfer (if and when it occurs) between different species of bacteria allows for new genetic combinations that can form novel genotypes, which may enhance invasiveness.

ACKNOWLEDGEMENTS

This work was funded primarily by DST-NRF Centre of Excellence for Invasion Biology (C•I•B) and the Working for Water Programme through their collaborative research project on 'Research for Integrated Management of Invasive Alien Species'. Additional funding was supplied by grants to D.M.R. from the National Research Foundation and the Hans Sigrist Foundation. J.J.LeR. also acknowledges the National Research Foundation for funding. The authors thank Pauline Ladiges and two anonymous referees for helpful comments on previous drafts of the manuscript.

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Editor: Pauline Ladiges